Genome-wide association mapping of Fusarium head blight resistance in contemporary barley breeding germplasm

J. Massman · B. Cooper · R. Horsley ·
S. Neate · R. Dill-Macky · S. Chao · Y. Dong ·
P. Schwarz · G. J. Muehlbauer · K. P. Smith

Abstract Utilization of quantitative trait loci (QTL) identified in bi-parental mapping populations has had limited success for improving complex quantitative traits with low to moderate heritability. Association mapping in contemporary breeding germplasm may lead to more effective marker strategies for crop improvement. To test this approach, we conducted association mapping of two complex traits with moderate heritability; Fusarium head blight (FHB) severity and the grain concentration of mycotoxin associated with disease, deoxynivalenol (DON). To map FHB resistance in barley, 768 breeding lines were evaluated in 2006 and 2007 in four locations. All lines were genotyped with 1,536 SNP markers and QTL were mapped using a mixed model that accounts for relatedness among lines. Average linkage disequilibrium within the breeding germplasm extended beyond 4 cM. Four QTL were identified for FHB severity and eight QTL were identified for the DON concentration in two independent sets of breeding lines. The QTL effects were small, explaining 1–3% of the phenotypic variation, as might be expected for complex polygenic traits. We show that using breeding germplasm to map QTL can complement bi-parental mapping studies by providing independent validation, mapping QTL with more precision, resolving questions of linkage and pleiotropy, and identifying genetic markers that can be applied immediately in crop improvement.

J. Massman · G. J. Muehlbauer · K. P. Smith (✉)
Department of Agronomy and Plant Genetics, University of Minnesota, 1991 Upper Buford Circle, Ste 411, Saint Paul, MN 55108, USA
e-mail: smith376@umn.edu

R. Dill-Macky · Y. Dong
Department of Plant Pathology, University of Minnesota, Saint Paul, MN 55108, USA

R. Horsley · P. Schwarz
Department of Plant Sciences, North Dakota State University, Fargo, ND 58105, USA

S. Neate
Department of Plant Pathology, North Dakota State University, Fargo, ND 58105, USA

B. Cooper
Busch Agricultural Resources LLC, Ft. Collins, CO, USA

S. Chao
USDA-ARS, Fargo, ND 58105, USA

Introduction

Since the advent and subsequent widespread utilization of molecular markers, plant geneticists have identified thousands of quantitative trait loci (QTL) in

bi-parental mapping populations (Bernardo 2008; Xu and Crouch 2008). However, the translation of that wealth of genetic information to plant improvement has been very limited (Bernardo 2008). When QTL information has been employed in marker-assisted selection (MAS), it has been primarily for traits that display simple inheritance or QTL that explain a substantial portion of the phenotypic variation. Often traits that could benefit most from MAS are those that are genetically complex and more intractable using traditional mapping approaches (Xu and Crouch 2008). Traits that are controlled by many genes with small effects, exhibit large epistatic effects, or are strongly influenced by the environment have generally not been improved through MAS. Thus, some of the most promising applications of molecular genetics approaches to plant improvement are yet to be realized.

Fusarium head blight (FHB) of barley, caused by *Fusarium graminearum* Schwabe [teleomorph *Gibberella* zeae (Schwein) Petch], is a classic example of a disease that has been extensively studied through QTL mapping, but has benefited little from MAS. FHB is a serious threat to agriculture worldwide and recent epidemics have led to intense breeding efforts to increase resistance to the disease and its associated mycotoxin, deoxynivalenol (DON) (McMullen et al. 1997; Bai and Shaner 2004). Resistance to FHB in barley is a complex quantitative trait, strongly influenced by environment, with low to moderate heritability (Van Sanford et al. 2001; Rudd et al. 2001; Capettini et al. 2003). Nine bi-parental mapping studies involving exotic sources of FHB resistance have identified many QTL for FHB severity in barley (Fig. 1). For many, but not all of these regions, QTL have been detected for DON concentration. Only a few of these QTL have been validated by subsequent studies (Mesfin et al. 2003; Canci et al. 2004) and currently only one is used in MAS (Nduulu et al. 2007). Identification of QTL for FHB has been confounded by agronomic traits that are correlated with disease severity, including plant height, heading date, and two- or six-rowed spike morphology (Zhu et al. 1999; Mesfin et al. 2003; Horsley et al. 2006). Co-segregation of these correlated traits confounds resistance QTL identification. Furthermore, it can be difficult to reveal the genetic architecture of these traits (linked QTL or pleiotropy) when multiple trait QTL are identified at the same locus. Two of the most

commonly identified QTL for disease resistance and reduced DON concentration are associated with spike morphology controlled by *vrs1* and a major heading date QTL, both on chromosome 2H (Fig. 1). In contrast to the mapping populations previously used to study FHB, breeding populations are generally more homogeneous for these correlated traits and therefore may be more effective for identifying QTL free of linkage drag or confounding effects.

Association mapping (AM) has recently gained popularity among plant geneticists as an alternative to overcome some of the limitations of bi-parental mapping (Jannink et al. 2001; Buntjer et al. 2005). One advantage is the ability to use existing germplasm collections and associated data sets, thus eliminating the need to generate new mapping populations from crosses among inbred parents. If AM is conducted within adapted germplasm, QTL can be identified and effects can be assessed in a more relevant genetic background. AM has been used successfully to identify QTL at both the candidate gene and whole genome levels within structured populations (Thornsberry et al. 2001; Zhao et al. 2007; Belo et al. 2008; Wang et al. 2008). In small grains, AM has been used in wheat to identify milling quality QTL (Breseghello and Sorrells 2006), and in barley to identify a number of trait QTL (Kraakman et al. 2004, 2006; Rostoks et al. 2006). These and other previous studies have used relatively small germplasm arrays that represent diversity in gene banks or regional cultivars and marker sets with limited genome coverage. However, AM has not yet been used to conduct genome-wide QTL mapping in existing breeding populations within a breeding pipeline. AM used in this way could help bridge the gap between QTL discovery and MAS in plant improvement and provide practical targets for genetic study.

Successful implementation of AM must consider the extent of linkage disequilibrium (LD) and population structure for the germplasm under study. The pattern of LD throughout the genome will determine the appropriate marker density for whole-genome scanning. While the extent of LD can vary widely depending on the population, in cultivated barley LD has been estimated to extend from 1 cM to beyond 10 cM (Kraakman et al. 2004; Caldwell et al. 2006; Malysheva-Otto et al. 2006; Rostoks et al. 2006). This range of values for the extent of LD is due, in
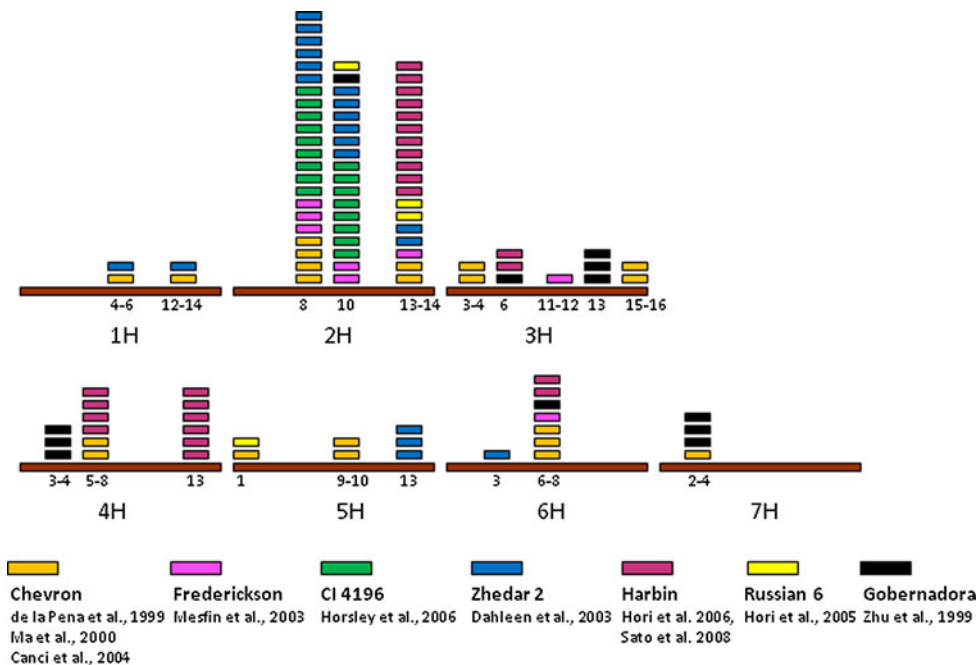
Fig. 1 Previously described Fusarium head blight QTL based on ten published studies. Each disk represents an environment in which a significant QTL was detected in that region of the chromosome. The *number* below the disks is the approximate bin position based on comparison of the markers included in the published study and the barley bin map (http://barleygenomics.wsu.edu/all-chr.pdf, checked 26 March 2009). The *different shades* correspond to different sources of resistance. Two major QTL for FHB severity are associated with a major heading date QTL (2H bin 8) and the *vrs1* locus (2H bin 10) that control two-row/six-row spike morphology

part, to the region of the genome studied, the number of loci investigated, and the reproductive history of the population under study. In general, LD appears to extend much further in elite breeding germplasm compared to wild populations (Caldwell et al. 2006). Differential relatedness among subgroups, or population structure, can inflate the number of spurious marker-trait associations identified through AM (Pritchard et al. 2000). Identifying and accounting for structure reduces the number of false positives (Yu et al. 2006; Zhao et al. 2007). A mixed model can be used for AM that accounts for population structure (*Q* matrix) as a fixed effect and differences in genetic relatedness (kinship or *K* matrix) as a random effect (Arbelbide et al. 2006; Yu et al. 2006).

In this study, we investigate the use of AM for FHB resistance, a trait with complex inheritance, using contemporary breeding germplasm. The objectives of this study were: (1) to assess phenotypic variation among breeding lines; (2) to characterize the extent of LD within the population; and (3) to map QTL for disease and DON mycotoxin resistance.

Materials and methods

Experimental design

The germplasm in this study consisted of 768 lines from advanced breeding populations from four breeding programs in the Upper Midwest that regularly evaluate FHB resistance. These lines have shown acceptable trait values for other important agronomic traits including plant height and heading date. Each program, Busch Agricultural Resources Inc. (BA), North Dakota State University two-row (N2), North Dakota State University six-row (N6), and the University of Minnesota (MN), contributed 96 lines in each of 2 years. The lines were inbred to at least the F4 generation and were selected to be representative of each program; not necessarily all lines submitted had FHB resistance. The variation for FHB resistance in the breeding programs is primarily due to the introduction of exotic parents. Thus the breeding lines evaluated represent a mixture of lines that have no exotic parentage and those that do. Lines with exotic

parents are from one to four breeding cycles away from the initial cross with the exotic parent. The lines were evaluated in multiple environments over 2 years. The first and second sets of 384 lines will be referred to as CAP I and CAP II, respectively.

To investigate various ways of conducting AM, we defined six mapping sets from the 768 lines in the study. The first two sets were the CAP I ($n = 384$) and CAP II ($n = 384$) sets described above. Splitting up the germplasm into CAP I sets and CAP II sets (which are related but independent) has the added benefit of assessing reproducibility of any given QTL region. The breeding programs develop both two-row (BA, N2) and six-row (BA, N6, MN) cultivars. Since previous studies have shown that two-row and six-row barley constitute distinct populations (Malysheva-Otto et al. 2006) and there are not many two-row by six-row crosses made within programs, four sets were defined by row type: CAP I six-row ($n = 224$), CAP I two-row ($n = 160$), CAP II six-row ($n = 243$), and CAP II two-row ($n = 142$).

Phenotypic evaluation

CAP I was evaluated at Crookston, MN, Fargo, ND, and Langdon, ND in 2006 and in Crookston, MN in 2007. CAP II was evaluated in Crookston, MN, Fargo, ND, Langdon, ND, and St. Paul, MN all in 2007. In each environment, the lines were planted into a randomized complete block design with two replications, and three common checks (Stander, Robust, and MNBrite). Inoculations were made using a grain spawn method (Horsley et al. 2006) in all environments except St. Paul, which used a conidial spray method (Steffenson 2003). Inoculum for North Dakota environments was produced using five isolates (KB-172, KB-176, KB-672, KB-582, and KB-173) applied at 28.0 g m^{-2} approximately 2 weeks and again 1 week before flowering. Inoculum for Minnesota environments was produced using local isolates collected from fields in Minnesota. In Crookston, grain spawn inoculum was applied at 5.6 g m^{-2} approximately 2 weeks before flowering and once again 1 week later. Two spray inoculations were made in St. Paul, MN. The first application occurred after heading, when greater than 90% of the spikes in a row were emerged from the boot, and the second application occurred 3 days later. Overhead mist irrigation was used in each environment to encourage disease development. Disease was

assessed by arbitrarily selecting ten spikes in each plot and visually estimating the percentage of kernels displaying symptoms. Mycotoxin content was assessed on harvested grain samples using gas chromatography and mass spectrometry (Fuentes et al. 2005; Mirocha et al. 1998). In Crookston 2006, Crookston 2007, and St. Paul 2007, plant height (PH) and heading date (HD) were assessed. PH was measured as the distance from the ground to the tip of the spike excluding awns, and HD was measured as the number of days after planting in which at least 50% of the spikes in a row were emerged at least half way from the boot.

Genotypic evaluation

DNA was isolated from a single plant from the bulk seed used in the phenotypic evaluation. Since all of the bulk seed was selfed to at least the F4 generation, single plants used for DNA extraction were at least F5. Tissue was freeze-dried at the University of Minnesota, and then sent to the USDA genotyping center in Fargo, ND. DNA was extracted using the method of Slotta et al. (2008). The DNA was genotyped using 1,536 single nucleotide polymorphism (SNP) markers. The SNPs were designed from EST sequences, and were organized into an Oligo Pool Assay (OPA). This set of markers is known as Barley OPA 1 (BOPA1; Close et al. 2009). The Illumina BeadStation was used to genotype each line utilizing the GoldenGate assay, as described in Fan et al. (2006). All marker and phenotypic data used in this study are available at http://thehordeumtoolbox.org.

The mapped markers from BOPA1 have been shown to be of very high quality (Close et al. 2009). When BOPA1 was screened on the barley breeding lines in this study we observed the expected level of heterozygosity for F5 inbred lines. To eliminate any markers with potential errors in allele calls, we removed all markers and individuals with more than 10% missing data points. Each marker with less than 5% minor allele frequency was also excluded. These filtered genotypic data sets were created for each mapping set, and subsequently used for the analysis of only that set. SNP marker positions were identified on a consensus map by Wu et al. (2008).

Statistical analysis

Phenotypic summary statistics including means, standard deviations, as well as an analysis of variance

were generated using SAS (SAS Institute 2002). Heritability was calculated on an entry mean basis across locations for CAP I and CAP II. All statistics were generated for each of the six mapping sets independently.

In each mapping set, LD was characterized using HAPLOVIEW v4.0 (Barrett et al. 2005). All pair-wise comparisons between markers were made using r^2 as the measure of LD. The pair-wise r^2 values were exported to Microsoft Excel and plotted against the distance (in cM) between the two markers. A moving average with a period of 100 pair-wise data points was used to characterize the pattern of LD. To compare the extent of LD between mapping sets, the distance (in cM) at which the moving average dropped below $r^2 = 0.20$ was recorded. Haploview was also used to visualize LD along the chromosome as well as LD between markers linked to QTL.

Prior knowledge of the breeding populations suggested there would be significant population structure in at least some of the mapping sets. To decrease the number of false positives, this structure was accounted for in the models used to map QTL. The program STRUCTURE v2.2 (Pritchard et al. 2000) was used to estimate the number of subpopulations within each mapping set. Each mapping set was analyzed using filtered SNP data, the admixture setting with correlated allele frequencies, and a burn-in and run time of 10,000 with $K = 1-7$ with five iterations. A subpopulation number was chosen by utilizing a number of factors, as proposed by Camus-Kulandaivelu et al. (2007). First, the ln(P) curve produced in Excel with data generated in STRUCTURE showed the probability of each subpopulation number. Secondly, STRUCTURE produces an assessment of inferred ancestry for each breeding line, which is a measure (in percent) of how much each line belongs to each subpopulation. The percentages were compared to prior knowledge about the lines to determine if the number of subpopulations partitioned the lines into their respective breeding programs. Finally, neighbor-joined dendrograms were generated using the software TASSEL v. 2.1 (Bradbury et al. 2007). Once a subpopulation number was determined, the inferred ancestry table was exported from STRUCTURE and used in the model for QTL mapping.

Mapping was conducted within each of the previously described six mapping sets. Association mapping was conducted using a mixed linear model analysis in TASSEL, utilizing scaled phenotypic data. For all mapping sets, each value at a location was divided by the standard error of that location to account for large differences in disease pressure. The association analysis used a mixed model (with the polygenic term as a random effect). The model used both the structure (Q) and kinship (K) matrices [see Yu et al. (2006) for a full description of the model]. Briefly, the Q matrix is the inferred ancestry output from STRUCTURE that accounts for coarse population structure. The K matrix is a measure of relative kinship and quantifies the probability that two homologous genes are identical by descent. The K matrix was generated within TASSEL utilizing the molecular marker data (Lynch and Ritland 1999).

One challenge with the large number of markers used in association mapping experiments is correcting for multiple hypothesis testing. To correct for multiple testing, we used a modified Bonferroni correction that incorporated the number of independent tests. The marker data in these mapping sets are not independent, in fact some markers have an $r^2 = 1.0$ and are redundant. Therefore, a SNP tagger in HAPLOVIEW was used to approximate the number of independent tests, and a Bonferroni correction was based on that number. Markers with an $r^2 \geq 0.8$ were considered to be not independent. The sets of independent markers were then counted to get a conservative estimate of the number of independent tests. The number of independent sets of markers was used in the Bonferroni correction to adjust the threshold to an experiment-wide significance level of $P = 0.05$.

The mixed model in TASSEL was used to identify markers associated with traits. The default run parameters were used, with the convergence criterion set at 1.0×10^{-4} and the maximum number of iterations set at 200. The $-\log(p)$ were plotted using R to visualize where significant markers were located across the genome (R Development Core Team 2008). Significant markers were declared using the modified Bonferroni correction at the $P = 0.05$ experiment-wide threshold. HAPLOVIEW was used to visualize the underlying LD at all candidate QTL regions, and assess the LD between pairs of candidate QTL both inter- and intra-chromosomally. Because there were large blocks of LD observed, many markers in the same region were significant. Linked

significant markers may be detecting the same QTL or linked independent QTL. To assess these possibilities, the LD between adjacent significant markers was used to define the bounds of a QTL. Significant markers were assigned to a QTL if there was less than a 5-cM gap between the significant marker and the most significant marker in the QTL region, or if the significant marker was less than 20 cM away, but in very high LD ($r^2 > 0.6$) with the markers in the QTL region. The length of the QTL region was then based on the map positions of all included markers.

Results

Phenotypic variation for disease resistance and correlated traits

In each environment, three checks varying in disease resistance were used to evaluate the disease nursery. These checks performed as expected, relative to each other, in every environment tested (data not shown). The heritability on an entry mean basis ranged from 0.38 to 0.61 for FHB severity and 0.55–0.76 for DON concentration across locations (Table 1). As expected, the heritabilities for HD and PH were generally higher, ranging from 0.56 to 0.75 for PH and from 0.82 to 0.95 for HD. There were significant ($P < 0.0001$) differences among lines for all traits (FHB, DON, PH, HD) at all locations (data not shown).

In each environment, the phenotypic correlation between the disease traits (FHB and DON) and the associated traits (PH and HD) was assessed. Within the six-row material PH was significantly negatively correlated with FHB severity in three experiments and with DON concentration in four experiments (r range: -0.23 to -0.54). Heading date was significantly positively correlated with DON concentration in two of the four experiments ($r = 0.14$ and 0.18), and negatively correlated with FHB severity in two of the four experiments ($r = -0.22$ and -0.34).

Marker coverage, LD, and population structure

Marker summary statistics are shown in Table 2. After the 1,536 SNPs were filtered for >5% minor allele frequency and <10% missing data there were 1,173, 564, and 1,113 SNPs in CAP I, CAP I six-row,

and CAP I two-row, respectively. The number of markers in the CAP II mapping sets was similar to CAP I. All mapping sets had good marker coverage with the average distance between marker loci ranging between 1.76 cM in CAP I to 3.19 cM in CAP I six-row. The CAP I six-row set also had the most gaps greater than 10 cM, with 19. The SNP tagger reduced the number of independent tests between 34 and 49% for the Bonferroni correction with the extremes being the CAP I two-row set and the CAP I six-row set, respectively. The average extent of LD varied between 4 and 8 cM among the different mapping sets. In general, the extent of LD was about twice as large in the six-row sets compared to the two row sets. We observed very little LD ($r^2 > 0.60$) between unlinked markers, although high LD between unlinked markers was more common in the mapping sets that included both two-row and six-row lines. Depending on the mapping set, about 60–70% of adjacent markers pairs were in LD with an r^2 value greater than 0.2 (Table 2).

Population structure was characterized using cluster analysis in STRUCTURE, a neighbor-joined dendrogram generated in TASSEL (data not shown), as well as prior knowledge about the germplasm. Generally, the different methods of characterizing structure provided similar results. Figure 2 shows the cluster analysis for CAP I. There was strong support for four subpopulations in CAP I that generally corresponded to the four breeding programs. The six-row breeding lines from the BA program had mixed membership to the subpopulations that corresponded to the MN and N6 programs. The two-row breeding lines in the BA program constituted their own group. We used the same approach to determine the correct sub-population number for each mapping set (data not shown).

Marker-trait associations

QTL for FHB severity and DON concentration were identified in each mapping set except for the two-row sets, which identified only QTL for DON concentration in the CAP I two-row set (Fig. 3). DON concentration QTL were identified on every chromosome, and FHB QTL were identified on every chromosome except 5H. Overall there were 37 regions identified for DON concentration and 14 for FHB resistance in at least one mapping set

Table 1 Phenotypic values and variation for Fusarium head blight (FHB), deoxynivalenol (DON), plant height (PH), and heading date (HD) within each mapping subset from 768 breeding lines

Set[a]	n	FHB (% symptomatic kernels)				DON (ppm)			
		Mean	SD	P[b]	H[c]	Mean	SD	P	H
CAP I	384	11.2	10.1	<0.0001	0.55	25.7	22.2	<0.0001	0.68
CAP I six-row	224	13.1	11.6	<0.0001	0.49	28.6	23.4	<0.0001	0.67
CAP I two-row	160	8.5	6.5	<0.0001	0.50	21.7	19.7	<0.0001	0.61
CAP II	384	6.7	5.7	<0.0001	0.39	15.4	10.3	<0.0001	0.72
CAP II six-row	241	6.6	6.0	<0.0001	0.61	17.1	11.3	<0.0001	0.76
CAP II two-row	143	6.9	5.3	<0.0001	0.38	12.4	7.6	<0.0001	0.55
	n	PH (cm)				HD (days after planting)			
		Mean	SD	P	H	Mean	SD	P	H
CAP I	384	81.7	10.7	<0.0001	0.64	52.1	3.4	<0.0001	0.90
CAP I six-row	224	83.3	11.5	<0.0001	0.73	51.6	3.1	<0.0001	0.82
CAP I two-row	160	79.6	9.0	<0.0001	0.75	52.7	3.7	<0.0001	0.91
CAP II	384	76.8	6.4	<0.0001	0.73	53.4	3.4	<0.0001	0.91
CAP II six-row	241	78.8	5.6	<0.0001	0.56	53.2	3.0	<0.0001	0.83
CAP II two-row	143	73.3	6.2	<0.0001	0.74	53.7	4.1	<0.0001	0.95

[a] Mapping sets which are subsets of 768 current barley breeding lines

[b] As determined from an analysis of variance across four locations

[c] Heritability determined on an entry mean basis across four locations

(Electronic Supplementary Material Table S1). The QTL identified were of small effect with markers ranging in r^2 from 0.01 to 0.05, and P-values as small as 5.00×10^{-15}. The boundaries of a QTL ranged in size from a single significant marker to markers spanning a 25 cM region (see DON6H.42-67 in Supplementary Table S1).

Using populations with complicated structure and kinship relationships to conduct AM raises the possibility of false-positive associations. To guard against that possibility, we included structure and kinship in the model and used a conservative threshold for detecting QTL. To focus on only the most robust and reproducible QTL, we further characterized only those QTL that were identified in both a CAP I and CAP II data set, based on the mean of four experiments. Using these criteria, eight QTL for DON concentration and four QTL for FHB severity were identified in both a CAP I and CAP II mapping set (Table 3). Marker r^2 values for these QTL ranged between 0.01 and 0.03 when estimated across locations and between 0.03 and 0.12 at individual locations. QTL were identified in as few as one environment and as many as six of the eight

tested environments. Two FHB QTL (FHB4H.24-36 and FHB6H.42-61) were coincident with QTL for DON (DON4H.21-36 and DON6H.42-67). The frequency of the resistance allele for each of the QTL was variable across the four breeding programs (Table 3). In general, the two-row program (N2) had higher frequencies of the resistance allele at FHB and DON QTL compared with the other programs. The coincident QTL, FHB6H.42-61 and DON6H.42-67, had the lowest resistant allele frequencies across all programs.

The two previously identified QTL regions for FHB and DON that have been most consistently detected on chromosome 2H bins 8 and 10 were both identified in this study. In the CAP I six-row set, DON2H.74-78 QTL was identified in chromosome 2H bin 8 in each environment (Fig. 4). A linked marker in this region (11_20528) was associated with HD in one of the two environments. The pattern of LD in this region shows that the markers associated with DON and HD are not in LD. This region corresponds to the Qrgz-2H-08 that has been previously mapped (Fig. 1). The DON2H.86 QTL was identified in the CAP I six-row set in chromosome 2H

Table 2 Characterization of the SNP data sets used for association mapping for six subsets of 768 breeding lines

Subsets[a]	n[b]	No. of SNPs[c]	No. of unique loci[d]	Average marker interval[e] (cM)	No. of gaps >10 cM	No. of Tag sites[f]	Average extent of LD[g] (cM)	Unlinked markers in high LD (%)[h]	No. of adjacent SNPs with r^2 >0.2
CAP I	384	1,173	619	1.76	2	711	6	1.27	689 (58%)
CAP I six-row	224	564	341	3.19	19	290	8	0.05	368 (66%)
CAP I two-row	160	1,113	599	1.81	3	737	4	0.05	626 (57%)
CAP II	384	1,127	601	1.81	2	670	6	1.18	698 (62%)
CAP II six-row	241	614	381	2.85	15	317	7	0.01	425 (70%)
CAP II two-row	143	1,067	576	1.89	3	692	4	0.07	622 (59%)

[a] Subsets of 768 breeding lines from four breeding programs in the Upper Midwest. The different row types were analyzed together (CAP I, CAP II) and independently (CAP I six-row, CAP I two-row, CAP II six-row, CAP II two-row)

[b] Number of individuals in each mapping set

[c] Markers included in the mapping analysis have <10% missing data and >5% minor allele frequency

[d] The mapping resolution from the consensus map was not able to resolve all markers into independent loci, thus some markers appear at the same location

[e] Average distance between all pairs of adjacent markers (cM)

[f] A SNP tagger in Haploview was used to approximate the number of independent sites among all markers. An r^2 of 0.8 was used as a threshold for independence. Some markers that map to the same locus were segregating independently

[g] Calculated by making all pair-wise marker comparisons for LD across the genome, and evaluating the moving average of r^2 values. A threshold of $r^2 = 0.2$ was used for LD

[h] The percent of all pair-wise marker comparisons with $r^2 > 0.60$

Fig. 2 Population structure of the CAP I mapping subset. Probability, ln $P(D)$, as a function of the number of sub-populations (k) calculated with STRUCTURE. The *bar chart* shows the inferred ancestry of each line with $k = 4$ subpopulations. The *breeding lines* are ordered by breeding program: University of Minnesota six-row (MN), Busch Agriculture (BA), North Dakota State University six-row (N6) and two-row (N2). The first third of the *breeding lines* in the BA program are six-row and the last two-thirds are two-row
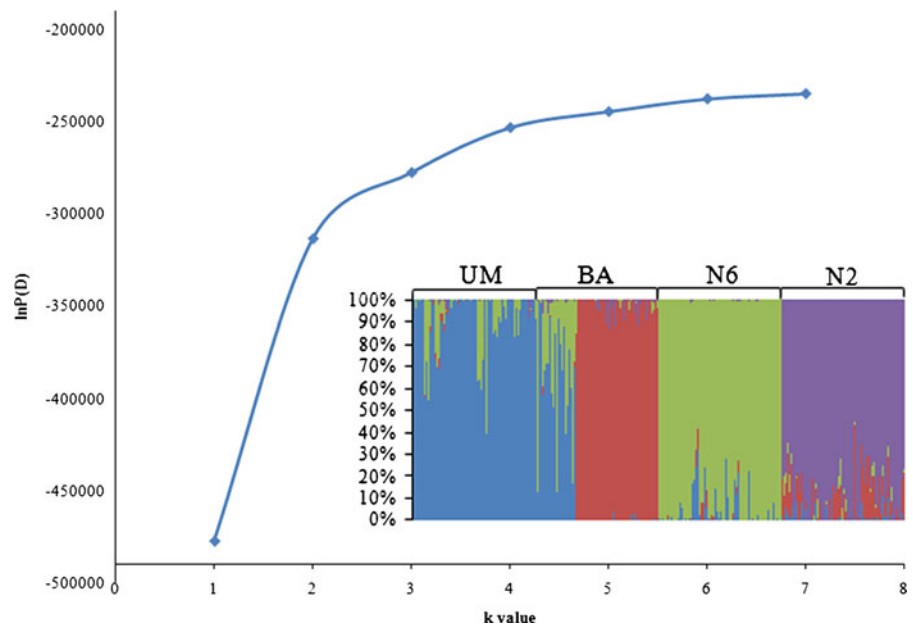
bin 10 in two of the four environments (Supplementary Table S1). This maps to the region of the previously identified Qrgz-2H-10 and is coincident with the *vrs1* locus.

Plant height and heading date were mapped for each individual environment for each set except CAP I and CAP II. In the CAP I two-row and CAP II two-row mapping sets there was one PH QTL

Fig. 3 *Genome scan showing* $-\log(p)$ *for marker associations with Fusarium head blight (FHB) severity and deoxynivalenol (DON) determined on 768* breeding lines *evaluated in four environments in 2006 and 2007.* Scans *are shown for the whole set of* breeding lines *and the six-row and two-row subsets for the CAP I and CAP II* breeding lines
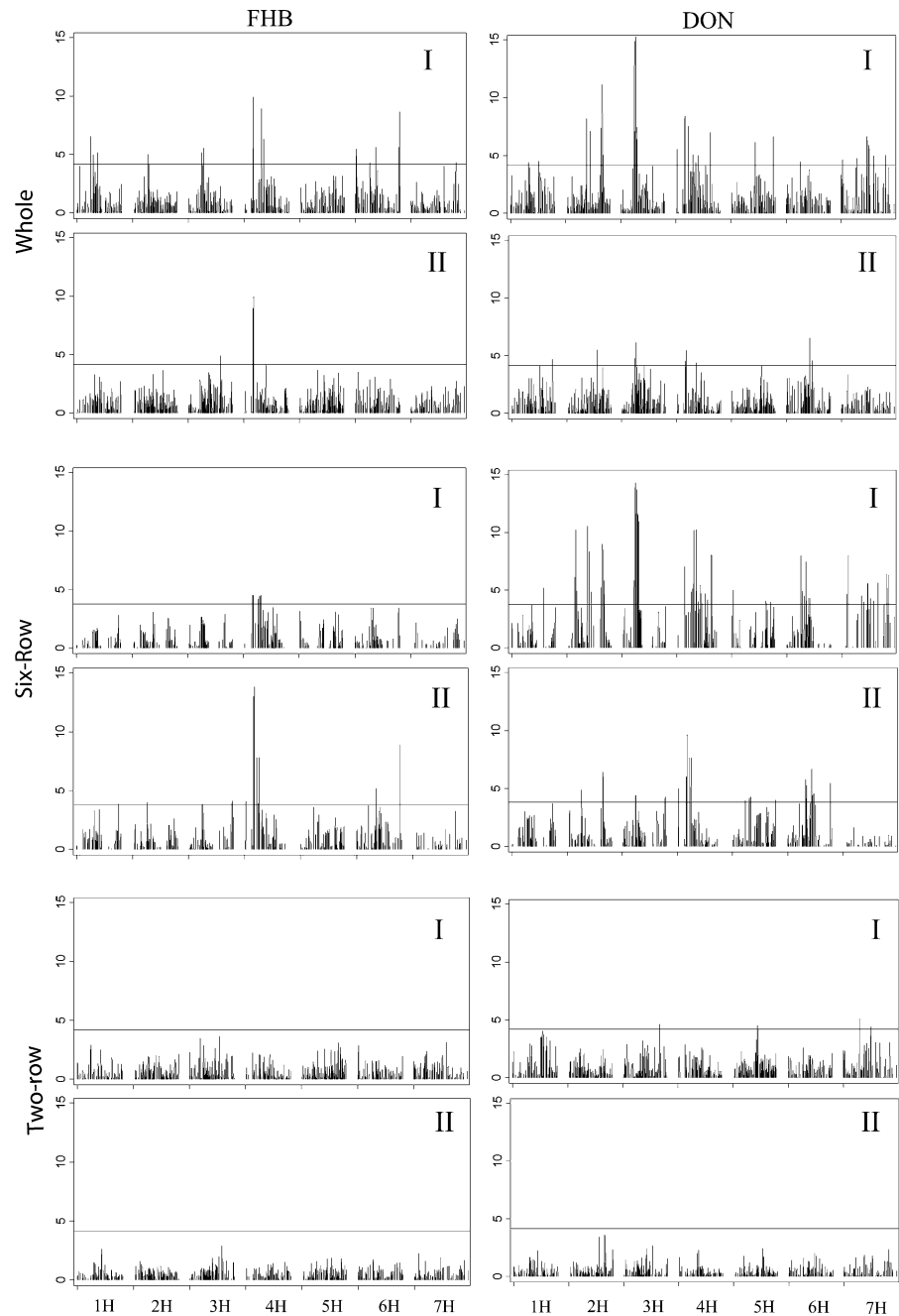
(chr. 7H cM 61.32) and one HD QTL (chr. 7H cM 38.3) identified. In the CAP I six-row and CAP II six-row mapping sets, eight genomic regions for PH and nine genomic regions for HD were identified in at least one environment (Supplementary Table S2). Three QTL regions for PH and one QTL region for HD were observed in both CAP I six-row and CAP II six-row.

There were five genomic regions identified that were significantly associated with at least one disease trait (FHB and/or DON) and PH or HD. Table 4 shows each coincident QTL including the traits, location, most significant marker, and P and r^2 values. DON and PH were coincident seven times (four genomic regions) over four experiments. Three of these regions were observed in two environments.

Table 3 Quantitative trait loci (QTL) associated with Fusarium head blight (FHB) severity and deoxynivalenol (DON) concentration identified in at least one mapping subset from CAP I and one mapping subset from CAP II, based on a mean of four experiments

QTL[a]	Bin[b]	r^2	No. of significant environments[c]		Resistant allele frequency within program[d]			
			Six-row (r^2 range)	Two-row (r^2 range)	MN	BA	N2	N6
FHB2H.50-56[d]	6–7	0.01	2 (0.04–0.05)	1 (0.07)	0.83	0.80	0.94	0.72
FHB4H.24-36	4–7	0.02	5 (0.04–0.12)	0	0.36	0.69	0.99	0.56
FHB6H.42-61	5–7	0.01	4 (0.03–0.09)	1 (0.05)	0.03	0.06	0.79	0.14
FHB6H.124-127	14	0.02	2 (0.04–0.06)	0	0.86	0.73	0.94	0.29
DON1H.88	9–12	0.01	nd[e]	0	1.00	0.90	0.74	0.99
DON2H.125-132	11–13	0.02	6 (0.04–0.07)	0	0.54	0.55	0.64	0.12
DON3H.52-65	4–7	0.03	4 (0.06–0.10)	0	0.19	0.57	0.95	0.0
DON4H.03	1	0.01	1 (0.04)	0	0.17	0.49	0.98	0.02
DON4H.21-36	2–5	0.02	6 (0.03–0.11)	0	0.33	0.58	0.99	0.02
DON4H.40-61	5–7	0.01	4 (0.05–0.09)	0	0.29	0.32	0.47	0.51
DON5H.190-192	13–15	0.01	1 (0.05)	0	0.85	0.81	0.95	0.54
DON6H.42-67	5–7	0.01	6 (0.05–0.12)	1 (0.09)	0.02	0.36	0.73	0.10

[a] QTL are named by the trait and chromosome. After the period is the position of the QTL in cM

[b] Bin location of flanking markers as determined from Oregon-Wolfe Barley mapping population (Syucs et al., *The Plant Genome*, accepted)

[c] QTL were mapped for CAP I six-row, CAP II six-row, CAP I two-row, and CAP II two-row within each individual environment to get the total number of significant environments for each QTL region (out of 8 total environments for six-row FHB and 7 for six-row DON and two-row FHB and DON)

[d] Frequency of most significant marker allele in CAPI

[e] Not determined. There were no markers with >5% minor allele frequency in this region in either CAP I six-row or CAP II six-row. This QTL was significant when mapped using CAP1 and CAPII (see Supplementary Table S1)

FHB and PH were coincident on 4H in two of four environments.

Discussion

QTL detection using contemporary breeding germplasm

In this study, we conducted AM using contemporary breeding lines and identified eight QTL for DON concentration and four QTL for FHB severity using a fairly conservative modified Bonferroni correction. These QTL regions were identified in two independent sets of breeding lines (CAP I and CAP II mapping sets). Four of the eight DON QTL regions identified in this study have been previously documented: DON1H.88, DON2H.125-132, DON4H.21-36, and DON4H.40-61 (de la Peña et al. 1999; Zhu

et al. 1999; Ma et al. 2000; Mesfin et al. 2003). Two of the four FHB QTL regions identified in this study (FHB4H.24-36, and FHB4H.42-61) have also been previously documented (Zhu et al. 1999; Sato et al. 2008). Thus, AM in breeding germplasm is capable of identifying both new and previously described regions associated with disease resistance.

One potential limitation using breeding populations for QTL mapping is less phenotypic variation than is typically present in populations derived from diverse parents, which could reduce the power to detect QTL. In this study, we found highly significant variation for all traits. The range of phenotypic values observed was considerably smaller in the breeding material compared to the wide-crosses used in previous bi-parental mapping studies. On average, the phenotypic ranges observed in the breeding material were approximately 60% of the ranges observed in wide-crosses (de la Peña et al. 1999;
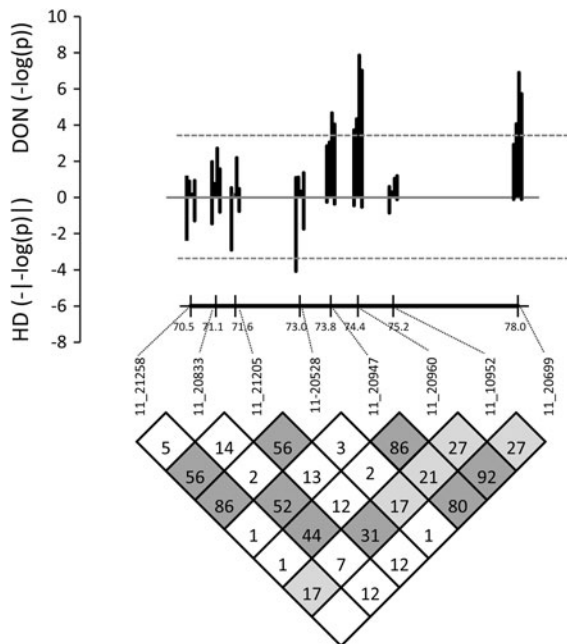
Fig. 4 Level of significance, log(p), and underlying linkage disequilibrium (LD) for eight SNP markers on chromosome 2H around the DON2H.74-78 QTL identified in the CAP I six-row mapping subset. Deoxynivalenol concentration (DON) was assessed in four locations, and heading date (HD) was assessed in two locations. *Numbers* above the map are the position of the markers from the concensus map in cM. LD is displayed in the *squares* below the map as r^2, expressed as a percentage, between all pair-wise combinations of the eight markers. *Light shading $r^2 < 15$, medium shading $15 < r^2 < 30$, dark shading $r^2 > 30$*

Ma et al. 2000; Dahleen et al. 2003; Mesfin et al. 2003; Horsley et al. 2006). The phenotypic range for FHB and DON in the CAP II mapping sets were less than in the CAP I sets, and were less than 50% of the wide-cross range. This may explain, in part, why fewer QTL for FHB and DON were identified in CAP II (24) than in CAP I (39) mapping sets.

Disease QTL explain small percent of phenotypic variation

Each QTL identified in this study explained less than 13% of the phenotypic variation for FHB and DON when estimated at individual locations. This is contrary to previous FHB mapping studies using wide-crosses where QTL with r^2 values as high as 0.60 have been identified (Horsley et al. 2006). Estimates of QTL effects were lower within the complete CAP I and CAP II sets. This is probably due

Table 4 Quantitative trait loci (QTL) associated with heading date (HD) and plant height (PH) that were coincident with either Fusarium head blight (FHB) severity or deoxynivalenol (DON) concentration QTL

Env[a]	CAP[b]	QTL[c]	P	r^{2d}	LD[e]	Pheno r^f
CR06	I	DON2H.74-78	2.39E-04	0.04		
		HD2H.73	1.07E-04	0.05	0.02	0.18
CR06	I	DON2H.125-132	2.32E-06	0.06		
		PH2H.126-132	1.84E-04	0.03	0.98	−0.54
SP07	II	DON2H.125-132	4.08E-05	0.05		
		PH2H.126-132	5.18E-05	0.04	1	−0.23
CR07	I	DON3H.52-65	5.71E-06	0.07		
		PH3H52-65	1.54E-06	0.06	0.14	−0.43
CR06	I	DON3H.52-65	1.28E-07	0.07		
		PH3H.52-65	1.04E-08	0.08	1	−0.54
CR07	II	FHB4H.24-36	2.59E-05	0.05		
		PH4H.24-27	5.76E-05	0.04	1	−0.35
SP07	II	FHB4H.24-36	5.22E-09	0.12		
		DON4H.21-36	2.04E-09	0.11	1	0.59
		PH4H.24-27	1.38E-05	0.04	0.75	n.s.
CR06	I	DON6H.42-67	2.55E-05	0.05		
		HD6H.43	5.35E-05	0.06	0.04	0.18
		PH6H.42-53	1.50E-07	0.06	1	−0.54
CR07	II	DON6H.42-67	4.15E-06	0.06		
		PH6H.42-53	3.18E-05	0.05	0.05	−0.26

[a] Environment; 06 = 2006, 07 = 2007; CR = Crookston, MN; SP = St. Paul, MN

[b] The 768 CAP breeding lines from four breeding programs in the Upper Midwest were divided into two subsets (I and II) of 384 lines and evaluated each year. Only six-row material (CAP I six-row, CAP II six-row) was used to identify coincident traits

[c] QTL names include the trait followed by the chromosome and centimorgan position of the QTL range

[d] Correlation between the most significant marker in the QTL region and the phenotype

[e] LD (r^2) was assessed between the most significant markers for each trait. When more than two QTL were identified in an environment, the LD presented is between the top QTL and the other two

[f] Correlation coefficient for the phenotypic data for the two traits from the environment the QTL coincidence was observed

to a number of reasons. First, with larger population sizes estimates of QTL effects are more accurate, whereas with small population sizes QTL effects are often biased upward (Beavis 1998; Melchinger et al. 1998). The identification of many QTL with small effects is consistent with the model of complex traits that predicts an exponential decay of QTL effects with very few QTL having large effects (Robertson

1967). Secondly, mapping within breeding germplasm, particularly for a trait under selection, may limit the ability to detect large-effect QTL. Under strong, multigenerational selection in a breeding program, large-effect QTL would be expected to become fixed more rapidly. In contrast, bi-parental mapping utilizes phenotypically diverse parents, and progeny populations with allele frequencies close to 0.5, and is expected to be most effective at identifying large-effect QTL. Furthermore, if a QTL is segregating or expressed in only one of the row types (six-row or two-row), then in analyses in larger data sets including both row types (CAP I and CAP II) the QTL would appear to have a smaller effect. Lastly, the effect of the marker is a function of the effect of the QTL and the LD between the marker and the QTL and insufficient marker density could lead to markers that are in low LD with the QTL.

If the relatively small r^2 estimates are accurate for the QTL we identified, then this has important implications for application of MAS. In contrast to large-effect QTL that are both easier to identify and maintain in breeding populations through phenotypic selection, the small-effect QTL identified in this study are more likely to be lost from breeding populations without the use of markers. Thus, MAS strategies may be necessary to accumulate and maintain many of the small-effect QTL to achieve an acceptable level of resistance within breeding populations.

Patterns of LD affect mapping resolution and false discovery

The QTL regions we identified varied substantially in size. There are a number of factors that influence the resolution of association mapping studies including population size, marker density, minor allele frequency, and the extent of LD within the target region. Within each mapping set, the latter three factors were variable across the genome. Across the mapping sets, each factor was variable both across the genome and in the same genomic region across different sets. For example, in this study, LD generally extended further in the centromeric region of 3H than it did towards the distal end of 2HL (data not shown). These regional differences in LD may explain, in part, why the QTL region for DON3H.52-65 is larger than that for DON2H.125-132 (13 and 7 cM, respectively).

While LD between markers and adjacent QTL allows for the identification of important genomic regions, LD between genetically distant markers may result in false discovery of QTL. Due to the high degree of relatedness within the mapping sets and the strong selection for FHB resistance, as well as other traits, it was not unreasonable to expect some QTL regions to be in LD with each other. Surprisingly, within each row-type set this was not the case, as little long-range LD between significant markers was observed. This was contrary to the CAP I and II sets, which included both two and six-row lines, where there was a substantial amount of long-range LD between significant markers (data not shown). This greater LD is likely due to population structure and differences in allele frequencies between subpopulations.

Marker density and extent of LD affect genome coverage

The number and distribution of SNP markers across the genome will affect the observed linkage disequilibrium, number of QTL detected, and estimated QTL effect size. The BOPA1 set of markers provides good coverage of the barley genome. When applied to the breeding lines in this study, many of the markers were not polymorphic. The gaps generated by monomorphic markers were likely due to regions of the genome that are fixed in this germplasm either as a result of selection or drift due to the limited number of parents that make up elite breeding populations. For those markers that were polymorphic in this germplasm, about 60–70% had adjacent marker r^2 values greater than 0.2. This suggests that there is a good chance of detecting QTL located between such markers. One of the clear tradeoffs between mapping in bi-parental populations and other germplasm arrays using association mapping is the marker density needed to guarantee complete genome coverage. Association mapping approaches will need to use more markers to accomplish similar coverage and because of the variable extent of LD across the genome will need high marker density in some regions. While we were able to identify many QTL, including several that were robust and validated in independent populations, it is possible that increasing the marker density would reveal even more QTL.

Population structure affects QTL detection

Because allelic diversity, allele frequency, and LD can differ among sub-populations, population structure can affect detection of QTL. Two-row and six-row barley are bred separately with relatively little crossing between the two groups. Genetic diversity studies in barley using molecular markers confirm that two-row and six-row types constitute distinct populations (Malysheva-Otto et al. 2006). We detected far fewer QTL for FHB severity and DON concentration in the two-row sub-population than in the six-row sub-population. The detection of fewer QTL within the two-row sets may be due to a number of factors including the amount of phenotypic variation, genetic background effects, the actual number of QTL segregating in the population, and population size. Phenotypic variation could affect the detection of marker-trait associations. While there were significant differences among lines in the two-row sets, the average phenotypic range was only about 60–80% of that in the six-row sets (data not shown). Differences in QTL expression among different genetic backgrounds have been previously documented for disease resistance in barley. Bilgic et al. (2005) identified different large-effect QTL ($r^2 > 0.35$) for spot blotch in different doubled haploid populations, which utilized the same source of resistance, but different two-row and six-row susceptible parents. Another possibility is that there are fewer QTL for FHB severity or DON concentration segregating within the two-row mapping sets. Breeding efforts in the Midwest to increase FHB resistance have intensified in the past 15 years. However, the use of exotic parents to introduce resistance genes has been more prominent in the six-row breeding programs. Finally, power to detect associations in two-row barley may have been lower as the population size was smaller for the two-row sets.

Mapping correlated traits

Mapping of traits that show a strong phenotypic correlation presents a significant challenge and raises issues of linkage versus pleiotropy. In the genetics of disease resistance, this question is particularly important as correlated traits can often affect the ability to accurately identify disease resistance QTL. One attractive feature of AM in breeding germplasm is the possibility that strong correlations often observed in wide crosses will be less so in breeding populations. In previous FHB studies, HD was negatively correlated with disease in ten and positively correlated in three out of twenty experiments (de la Peña et al. 1999; Ma et al. 2000; Dahleen et al. 2003; Mesfin et al. 2003; Hori et al. 2005). In our study, HD was both positively and negatively correlated with FHB and DON. The lack of consistent negative correlation may be due, in part, to more homogeneous heading dates in breeding populations. While there is often a negative correlation between disease and HD within bi-parental mapping studies, breeding and recombination may have broken the linkage between FHB and HD QTL, or selection may have resulted in discarding any individuals that had pleiotropic QTL.

Overall, within the six-row mapping sets, we observed less coincidence among HD or PH QTL and disease QTL than in previous bi-parental mapping studies. Within the six-row mapping sets, 38% (3 out of 8) FHB and 29% (8 out of 28) DON QTL were coincident with a HD or plant height QTL (Supplementary Table S1). This is less than published wide-crosses which found 43% (23 of 54) FHB and 46% (13 of 28) DON QTL coincident with PH or HD (de la Peña et al. 1999; Ma et al. 2000; Dahleen et al. 2003; Mesfin et al. 2003; Hori et al. 2005). Less coincidence may be due to the extent of phenotypic variation for the traits in question. In previous studies, the range of HD in bi-parental mapping populations was 12–20 days (Ma et al. 2000; Horsley et al. 2006). In this study, the range of HD was 7–14.5 days, with only one location having a range greater than 10 days. Less phenotypic variation for HD suggests that fewer HD QTL are segregating in breeding populations and, therefore, possibly fewer HD QTL are coincident with disease resistance. In our study, HD QTL were coincident with only 2 of 28 DON QTL and none of the FHB QTL. A reduction in unfavorable linkages may be a unique advantage to conducting association mapping within breeding populations. With less coincidence between disease and morphological trait QTL, there are not only fewer problems with linkage drag, but also less likelihood that the correlated trait will confound the identification of disease QTL.

Evidence for linkage rather than pleiotropy at two disease resistance QTL

Two of the most commonly identified QTL for FHB severity in barley are coincident with other traits (HD and row type). We observed coincident QTL for DON concentration and HD 13 cM proximal to *vrs1* on chromosome 2H (Fig. 4). This is likely the large-effect FHB and DON QTL region (Qrgz-2H-8) in bin 8 of 2H that has been identified in multiple studies (Fig. 1; de la Peña et al. 1999; Mesfin et al. 2003; Horsley et al. 2006). At the same location is a large-effect HD gene, which may have a pleiotropic effect on resistance or may be linked to a resistance QTL (Canci et al. 2004). Delayed head emergence may increase the likelihood that the host will escape infection by the pathogen. Nduulu et al. (2007) concluded, using near-isogenic lines, that FHB and HD on 2H were correlated due to tight linkage in the region. Our results are consistent with this interpretation as DON and HD QTL were mapped in the Qrgz-2H-8 region and were identified with distinct markers 1.4 cM apart (Fig. 4). Low LD ($r^2 = 0.02$) between the two markers (11_20528 and 11_20960) indicates the markers are segregating almost independently, which lends evidence to linkage, rather than pleiotropy, as the explanation for the correlation between these two traits.

The other well-documented region associated with FHB and DON is located on chromosome 2H bin 10 and referred to as Qrgz-2H-10 (Fig. 1; Ma et al. 2000; Dahleen et al. 2003; Mesfin et al. 2003; Horsley et al. 2006). This important resistance QTL is either linked to the *vrs1* (row-type) gene or is a pleiotropic effect of this locus. One possibility is that the more open architecture of the two-row spike provides a less favorable environment for disease to develop compared to the six-row spike. Previous work has been unable to make definitive conclusions about this QTL region. Ma et al. (2000) crossed two-six-row barleys and identified a FHB QTL on chromosome 2H bin 9–15. Unfortunately, this wide QTL region covered two previously described QTL regions (bin 10 and bin 13–14) and is near another (bin 8). Hori et al. (2006) crossed two–two-row parents and did not identify the 2H bin 10 region as significant, and concluded that the same resistance allele was present in each parent. Sato et al. (2008) also crossed among two-rowed parents and also failed to identify the 2H bin 10

region. Mesfin et al. (2003) and Horsley et al. (2006) each identified a QTL for FHB and DON coincident with *vrs1* within two-row by six-row populations. Neither study could unambiguously distinguish between pleiotropy and linkage. In our study, we identified a DON QTL at the same region as *vrs1* in both CAP I and CAP I six-row. Identification of this locus within the six-row mapping subset, where *vrs1* is fixed, provides evidence that the two traits are under separate genetic control.

Conclusion

AM directly in contemporary breeding germplasm has the potential to accelerate the translation of basic genetic information towards application in plant improvement. The availability of high density DNA marker platforms in important crop species has created the opportunity to conduct genome-wide scans for QTL. Germplasm arrays other than bi-parental populations will be useful, however special attention will need to be paid to the number of markers needed to accomplish whole genome coverage relative to the extent and patterns of LD. Using breeding germplasm to map QTL can complement bi-parental mapping studies by providing independent validation, potentially more precise QTL locations, and evidence which might point towards linkage or pleiotropy. Furthermore, using breeding populations creates the opportunity to use existing data sets from large numbers of lines and increase the power to detect QTL with small effects. The QTL identified in this study are in many cases free of undesirable linkages and immediately available for the development of MAS strategies. Understanding the distribution of resistance alleles in a breeding population will allow breeders to make crosses among elite lines that contain complementary desirable alleles. Considering the relatively large number and small effects of the QTL identified here, a more comprehensive marker selection strategy, such as genomic selection (Heffner et al. 2009), may be more effective than traditional marker strategies at accumulating beneficial alleles in breeding populations.

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